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Raphidophyceans on the coasts of Mexico

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Abstract

The presence of ichthyotoxic phytoflagellates *Chattonella marina*, *Fibrocapsa japonica*, and *Heterosigma akashiwo* of the algal class Raphidophyceae are reported for first time in the Gulf of Mexico and the Pacific coast of Mexico. Phytoplankton were sampled to isolate and identify species, and to develop growth experiments in different media. We observed living material of the three species, but were unable to recognize specimens in preserved samples. Cultures were established by isolating single cells and diluting phytoplankton samples in modified $f/2$ and Erd–Schreiber media. Features, including morphological variations and pigment composition, of living cells under various nutrient conditions are described. These ichthyotoxic species produce harmful blooms in several parts of the world, although no documented cases have been reported in Mexico.

Introduction

The autotrophic flagellates of marine plankton, also called phytoflagellates, have not been studied adequately in Mexico. Cells are extremely fragile, and usually they are not recognized in preserved phytoplankton samples (Jeffrey & Vesk, 1997). Among these phytoflagellates are a small group of microalgae belonging to the class Raphidophyceae, within the division Heterokontophyta, composed of a few genera and species (Hallegraeff & Hara, 1995; Van den Hoek et al., 1995; Lee, 1999).

The most important characteristics of the Raphidophyceae are: solitary and motile monad cells, ovoid to elongate or pyriform in shape, absence of rigid coverings, 10-80 μm long, numerous yellow, golden, or sometimes green chloroplasts with two membranes of the endoplasmic reticulum, chlorophyll *a* and *c* present, and two heterodynamic flagella inserted in the anterior part of the cells. The anterior flagellum is directed forward, is dynamic, and has mastigonems. The other flagellum is directed towards the posterior, is smooth with no mastigonems, and is not dynamic (Heywood, 1980a; Hallegraeff & Hara, 1995; Van den Hoek et al., 1995; Lee, 1999). Raphidophyceae were reported as predominantly freshwater species (Heywood, 1980b), but coastal blooms of marine species have become increasingly common, sometimes causing serious damage to the mariculture industry (Jeffrey & Vesk, 1997).

Marine Raphydophyceae species *Heterosigma akashiwo* (Hada) Hada ex Hara et Chiahara, *Chattonella marina* (Subrahmanyan) Hara et Chihara, and *Fibrocapsa japonica* Toriumi et Takano have broad environmental tolerance and have been found mainly in tropical and temperate coastal waters. They are considered ichthyotoxic (Onoue & Nozawa, 1989; Shumway, 1990; Chang et al., 1990; Onoue et al., 1990; Yang et al., 1995; Khan et al., 1996; Tomas et al., 2001), and have caused severe fish mortalities with significant damage to the mariculture economy (Taylor, 1993; Okaichi, 1997; Tomas, 1998) in several countries (Chang et al., 1990; Taylor, 1990, 1993; Taylor & Horner, 1994; Hallegraeff et al., 1998;

Tiffany et al., 2001). Although the precise mechanism of the toxins produced by these organisms remains unclear, production of brevetoxins (Khan et al., 1995a, 1996, 1997), reactive oxygen species (ROS) (Ishimatsu et al., 1996), including hydrogen peroxide, superoxide anion, and hydroxyl radicals (Oda et al., 1994; Tanaka et al., 1994; Twiner & Trick, 2000; Kim et al., 2001), and highly unsaturated fatty acids (Okaichi, 1987) have been suggested as the main factors contributing to fish mortality by damaging gill tissues, leading to asphyxiation (Kim et al., 2001; Tiffany et al., 2001). Polyunsaturated fatty acids account for 46–50% of the total fatty acids of *C. antiqua* and *H. akashiwo* (Nichols et al., 1987).

Raphidophytes have been reported in the Western Hemisphere. *Heterosigma akashiwo* has been recorded on the east and west coasts of the United States, Canada, and Chile (Loeblich & Fine, 1977; Tomas, 1978; Haigh & Taylor, 1990; Taylor, 1990, 1993; Parra et al., 1991; Taylor & Haigh, 1993; Horner et al., 1997). *Chattonella marina* was associated with fish mortality in Salton Sea, California (Tiffany et al., 2001). However, it has not been associated with harmful effects in Florida (Tomas, 1998). Recently, the genus *Chattonella* has been recorded along the coast of Brazil (Moestrup, 2002). *Fibrocapsa japonica*, first described as *Botryococcus* sp. (Toriumi & Takano, 1973), has been recorded in samples from Belize (Moestrup, 2002) and at sites along the west and east coasts of the United States and Brazil (Tomas et al., 2001). These species had not been reported in Mexican coastal waters, and no massive blooms, fish mortalities, loss of marine resources, or human poisoning have been associated with these species. This study describes the influence of different nutrient conditions on growth and morphology of vegetative cells and pigment composition of Raphidophyceans isolated from different localities of Mexican coasts.

Material and methods

Phytoplankton samples were collected from Bahía Concepción, Mazatlán, and Acapulco along the Pacific, and from Tuxpan on the Gulf of Mexico (Fig. 1) during 1997, 1998, and 2000. Phytoplankton was collected by vertical tows, using plankton nets of 20- and 54-μm mesh, and from the surface to 5 m in 5-l Van Dorn bottles. Phytoplankton cells concentrated by plankton tows were sieved through a 60-μm mesh to eliminate larger organisms, and inoculated in a 250-ml culture container with sterilized seawater enriched with $f/2$ medium modified by adding H_2SeO_3 (10^{-8} M) and reducing the concentration of $CuSO_4$ to 10^{-8} M (Anderson et al., 1984). Seawater collected from the surface was filtered by gravity, concentrating plankton (<1.2-μm size) into approximately 50 ml of media. This material was divided into: (a) a reference sample fixed with acid lugol, (b) material for immediate observation by light microscopy (Zeiss, Axiolab), and (c) an inoculum for establishing cultures.

Vegetative cells were isolated with micropipettes under an inverted microscope (Carl Zeiss, Axiovert 100). The cells were transferred individually to 96-well assay plates, previously filled with modified $f/2$ medium, and maintained at 21 ± 1 °C, L: D, 12:12 with 150 μmol m^{-2} s^{-1} light intensity. Culture media were prepared using aged Bahía Concepción seawater (salinity ~35). Seawater and nutrients were sterilized through a 0.22-μm filter. Cultures from wells were transferred into 75-ml culture flasks for experiments and identification. A fraction of the material concentrated by gravity was injected into 5-ml assay tubes and 250-ml culture flasks with Erd–Schreiber medium (Throndsen, 1997). Culture room conditions were the same as described above.

Observations of cells under light microscopy were recorded with a digital camera (Cool Snap-Pro, Media Cybernetics). Identification was based on Hallegraeff & Hara (1995) and Throndsen (1997) and on morphological characteristics, such as cell size and shape, number, color, and shape of chloroplasts, flagella type and movement, presence of mucocysts, and nucleus and pyrenoid locations.

All experiments on vegetative cells were based on single non-axenic strains that could be maintained for a long period, such as *F. japonica* (FJCV-1) and *C. marina* (CMCV-1), which were isolated from Bahía Concepción in 2000. These species were grown in triplicate 25-ml tissue culture flasks with: (a) GSe medium (Blackburn et al., 1989); (b) modified $f/2$ (Anderson et al., 1984); (c) $f/2$ nitrate deficient medium; and (d) $f/2$ phosphorus-deficient medium. All other major nutrients, trace metals, and vitamins were supplied at $f/2$ concentrations at 20 °C with overhead illumination by white fluorescent lights at conditions specified above. Assay plates were checked every third day with an inverted microscope to observe and photograph the sequence of morphological changes. After 14 days, 2 ml of each culture was fixed with an acid lugol solution, and dispensed on 1-ml Sedgwick-Rafter slides for quantification. Student's *t*-test was used to de-

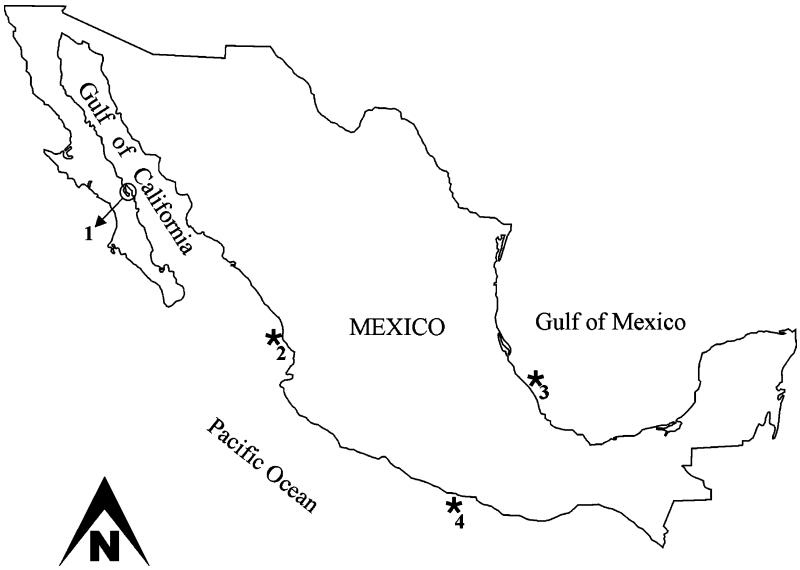


Figure 1. Location of collection sites: (1) Bahía Concepción, (2) Mazatlán, (3) Tuxpan, (4) Acapulco.

termine significant differences among maximum cell densities in different media.

For pigment analyses, 25 ml of each culture was filtered through GF/F glass fiber and frozen immediately at −20 °C. Pigments were extracted and injected into an HPLC system (Series 1100, Hewlett Packard), under conditions described by Bustillos-Guzmán et al. (2000).

Results

Heterosigma akashiwo (Hada) Hada ex Sournia (Figs 2, 3)

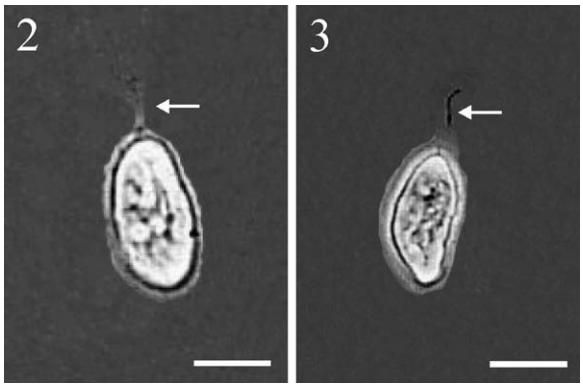
Basionym: *Entomosigma akashiwo* Hada.

Synonyms: *Heterosigma inlandica* Hada, *Chattonella inlandica* (Hada) Loeblich III et Fine, *Olisthodiscus luteus* N. Carter, *Olisthodiscus carterae* Hulburt, *Chattonella akashiwo* (Hada) Loeblich III, *Heterosigma carterae* (Hulburt) F. J. R. Taylor.

References: Taylor, 1992; Tomas, 1979; Hara & Chihara, 1987, p. 153, Figs 1–10, 21; Parra et al., 1991, p. 102, Figs 1–5; Hallegraeff & Hara, 1995, p. 365, Fig. 18.1; Itakura et al., 1996, p. 1976, Fig. 1; Throndsen, 1996, p. 367; Horner et al., 1997; Khan et al., 1997; Throndsen, 1997, p. 616, Plate 2; Connell, 2000.

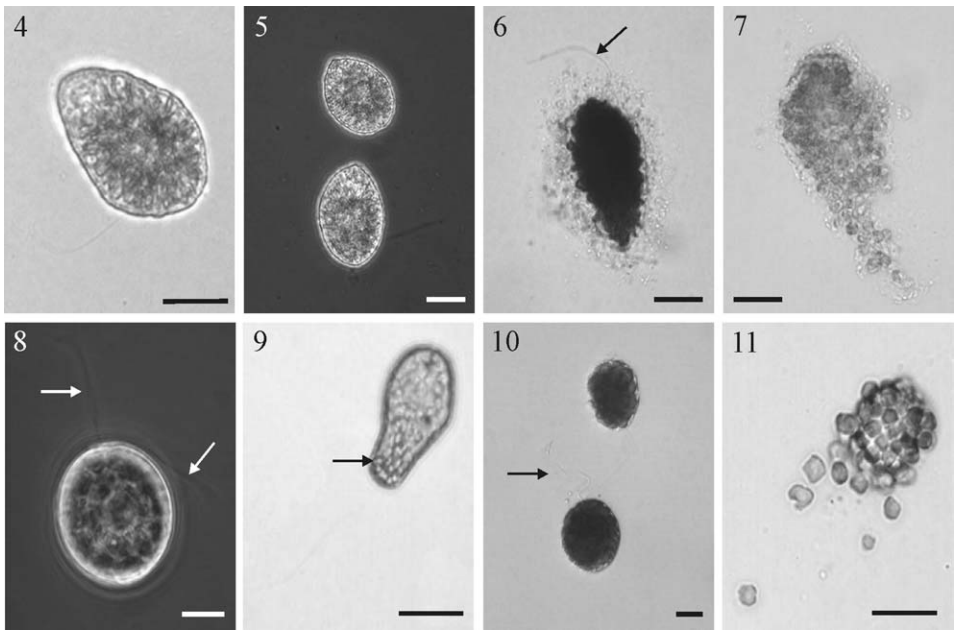
Type locality: Seto-Naikai Inland Sea, Japan.

Description: Cells had variable shapes, from subspherical to ovoid, or oblong or slightly elongate, and compressed dorso-ventrally (Figs 2, 3). Dimensions 12–18 μm wide and 18–28 μm long. Cell periphery had 8 to 15 discoid chloroplasts, brown or yellow-brown in color. There were two flagella emerging from the anterior of cells, one of which appeared dynamic, and the other almost rigid. The drop-shaped nucleus was conspicuous and located in the central part of each cell. Cells swim in a spiral pattern, interrupted only by other organisms or elements in their way. When this happened, cells back up slightly and continue on their way in another direction. No resting cells or stages were found.



Figures 2–3. Heterosigma akashiwo. Scale bars = 10 μm. Large cells showing dynamic flagellum (arrows), and various chloroplasts.

This species commonly appeared at Mazatlán, Acapulco, and Tuxpan, but was never abundant, and



Figures 4–11. Live and fixed cells of *Chattonella marina* (4–7) and *Fibrocapsa japonica* (8–11). Scale bars = 20 μm. Live cells (4, 5), acid lugol (6) and formalin (3%) fixed cells of *C. marina* (7). Live cell of *F. japonica* showing flagella (8) and trichocysts (9), acid lugol (10) and formalin (3%) fixed cells of *F. japonica* (11).

there was no evidence of massive blooms. No trace of this species was found in preserved samples.

Chattonella marina (Subrahmanyan) Hara et Chihara (Figs 4–7, 12–33)

Basionym: *Hornellia marina* Subrahmanyan.
References: Hallegraeff & Hara, 1995, p. 368, Fig. 18.6; Khan et al., 1998; Ono et al., 1998; Tiffany et al., 2001, p. 189, Figs 1 a–e.

Type locality: Malabar Coast, southwest India.

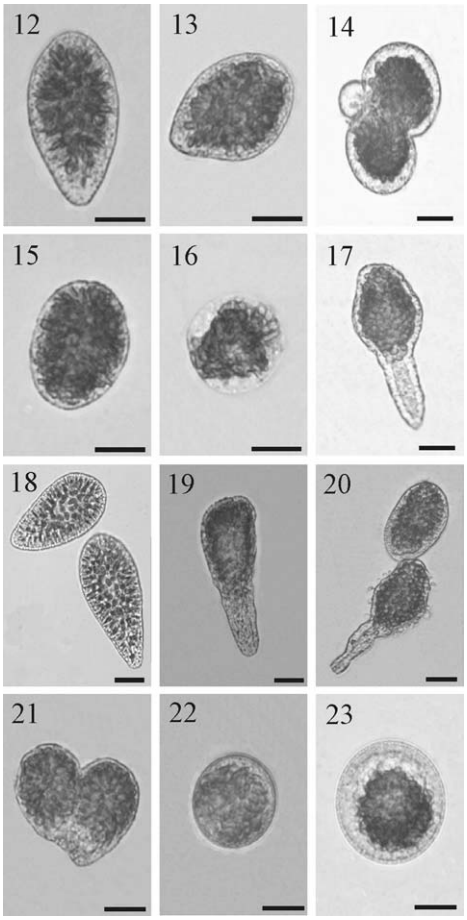
Description: Motile cells were almost twice as long as wide (62–77 μm × 35–37μm), with two heterodynamic flagella situated in a small apical depression (Figs. 4–6, 12, 13, 15, 18, 19, 24–26, 29–32). Cells fixed with acid lugol solution maintain the general shape of the living cells and some retain the flagella (Fig. 6), in contrast to cells preserved in 3% formalin, which disintegrate (Fig. 7). Morphology of *C. marina* is influenced by the age of the culture and media. As cultures age, cells became more ovoid and spherical non-motile cells (11–38 μm) appear (Figs 15, 16, 22, 23, 27, 28, 33). Asexual reproduction occurs by binary fission while cells are swimming (Fig. 21). Some amorphous cells were observed in $f/2$ media after nine days of growth (Fig. 20). In GSe media, some cells appeared narrower at the anti-apical end of the cell (Fig. 17). In $f/2$ N-limited media, cells were narrower (21–31 μm) and longer (32–86 μm) than in GSe media. Oval cells in $f/2$ N-limited cultures were smaller (21–22 μm), and cultures have less pigmentation than in GSe and $f/2$ P-limited media (34–38 μm) (Figs 24–28). Morphology of cells preserved with acid lugol solution change, but cells could be recognized for counting, if obtained from cultures.

Fibrocapsa japonica Toriumi et Takano (Figs 8–11,34–46)

Synonym: *Chattonella japonica* (Toriumi et Takano) Loeblich III et Fine; *Exuviella* sp. Iwasaki, *Olisthodiscus luteus* Carter, *Botryococcus* sp.
References: Toriumi & Takano, 1973; Hallegraeff & Hara, 1995, p. 366, Fig. 18.3; Throndsen, 1997, p. 616, Plate 2; Tomas et al., 2001, Fig. 4.

Type locality: Atsumi Bay, Japan.

Description: Cells usually longer than wide (21–33 μm × 19–24 μm), with two apical flagella. The anterior flagellum is as long as the cell, and the posterior is 1.2 times the cell length (Fig. 8). Chloroplasts are golden brown and densely packed in the cell. Trichocysts, the distinctive feature of this species, are almost absent in this strain, and only few cells showed this feature (Fig. 9). Cells fixed with acid lugol solu-



Figures 12–33. Chattonella marina grown in GSe (12–17), and $f/2$ (18–23) media. Scale bars = 20 μm. (12, 13) Spindle cells, (14) dividing cells, (15) oval cell, (16) spherical non-motile cell, (17) spindle cell, (18, 19) spindle cells, (20) amorphous and oval cell, (21) asexual division, (22, 23) spherical non-motile cells.

tion acquire a brambleberry form, and only a few cells retain the flagella (Fig. 10). As in *C. marina*, cells fixed with formalin (3%) disintegrate (Fig. 11). *Fibrocapsa japonica* also changed morphologically with culture age and growth media (Figs 35, 36, 43, 44, 46). In $f/2$ and $f/2$ P-limited media, cells were oval (Figs 40, 45). In GSe media and $f/2$ N-limited media, cells were pear-shaped and some were amorphous (Figs 34, 35, 43). Cells in $f/2$ N-limited medium were usually longer than cells in other media (Table 1). Non-motile cells (20–26 μm) were observed in $f/2$ P-limited and GSe medium cultures (Figs 35, 36, 46). Asexual reproduction occurred by binary fission (Figs 37–39). The morphology of cells preserved in acid lugol solution changed drastically, but cells could be recognized for cell count, if obtained from cultures.

After 9 days of culture, the lowest cell density ($P < 0.05$) in *C. marina* cultures was in phosphate-limited medium with 1328 ± 268 cells ml^{-1} (Fig. 47). In GSe, $f/2$, and $f/2$ N-limited media, cell density was higher (2783 to 3762 cells ml^{-1}) than in $f/2$ P-limited medium. *Fibrocapsa japonica* also showed the lowest cell density ($P < 0.05$) in $f/2$ P-limited medium (1870 ± 359 cells ml^{-1}), and in GSe, $f/2$, and $f/2$ N-limited media cell density varied from 3500 to 4139 cells ml^{-1}.

Chattonella marina and *F. japonica* had pigments typical of Raphidophyceae (Table 2). The most abundant pigment in both species, cultivated in $f/2$ or GSe medium, was chlorophyll *a* (61–68%), followed by fucoxanthin (22–25%), chlorophyll *c*1–*c*2 (7–9%), β-carotene (2–3%), and diadinoxanthine (0.3–4%). There was no difference in pigment compositions in *C. marina* and *F. japonica* when cultivated in either media. *C. marina* showed less diadinoxanthin in $f/2$ than in GSe medium (0.3% and 4%, respectively).

Discussion

Taxonomy of the Raphidophyceae has been based largely on morphological characteristics and on observations of wild or cultured organisms. Some ultrastructural studies have shown additional details, such as pyrenoids directed toward the inner cell, shape of nucleus, and the relationship of the nucleus to flagellar roots (Tomas, 1979; Hara et al., 1985; Hara & Chihara, 1987; Vesk & Moestrup, 1987).

There have been discussions about taxonomic placement of certain species of the group: *Chattonella subsalsa* Biecheler (the genus) is closely related to *C. marina*, and some authors (Hallegraeff & Hara, 1995; Tomas et al., 2002) have discussed possible co-specificity of the two species. Throndsen (1996) discussed the taxonomy of the species *H. akashiwo* following the rules of the International Botanical Nomenclature Code, but despite that, Taylor (1992) proposed a taxon that apparently fulfilled nomenclature requirements and gave priority to Hara & Chihara (1987), who provided a diagnosis and holotype of the species in Latin. Throndsen (1996) also discussed the confusion surrounding this species and the closely related species *Olithodiscus luteus* Carter, which has a benthic habitat and morphological differences (Hara et al., 1985).

A number of authors have observed morphological variations in cultured Raphidophyceae species

Table 1. Average cell measurements (μm) of *Chattonella marina* and *Fibrocapsa japonica* cultured in different media.

	GSe		$f/2$		$f/2$ (N-limited)		$f/2$ (P-limited)	
	Length	Width	Length	Width	Length	Width	Length	Width
C. marina	61.7	37.1	77.2	35.7	68.5	26.8	65.5	37.0
F. japonica	32.7	23.5	21.0	18.6	40.7	22.0	29.3	26.5

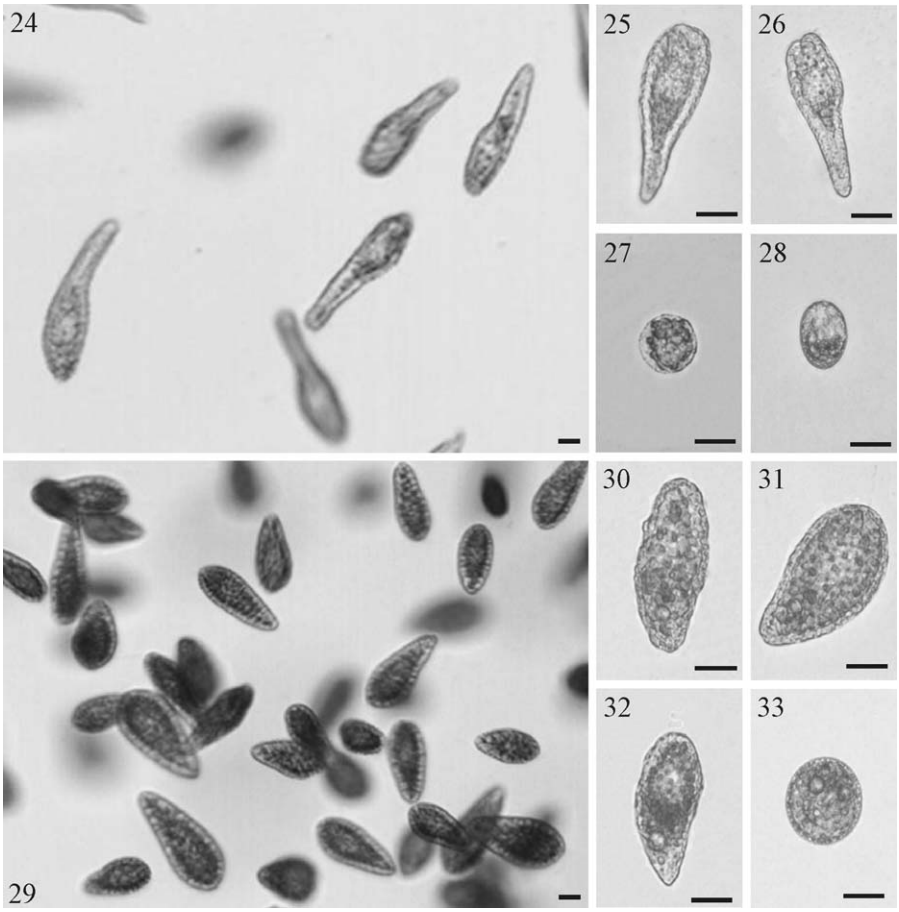
Table 2. Pigment composition (%) of *Chattonella marina* and *Fibrocapsa japonica* cultivated in $f/2$ and GSe media.

	Media	Chlorophyll *a*	Chlorophyll *c*	Fucoxanthin	Diadinoxanthin	β-carotene
C. marina	$f/2$	67.6	7.4	22.0	0.3	2.7
	Gse	61.5	9.0	23.2	4.2	2.1
F. japonica	$f/2$	62.3	9.1	25.4	1.4	1.9
	Gse	62.7	9.1	25.3	1.2	1.8

(Tomas, 1980; Parra et al., 1991; Aizdaicher, 1993; Connell & Cattolico, 1996). Nonmotile cells of *H. akashiwo* were recognized in sediments from Japanese waters (Imai & Itakura, 1991). Cell morphology of these species has been used as an indicator of culture health (Furuki et al., 1981; Nakamura & Watabe, 1983; Khan et al., 1995b; Marshall & Hallegraeff, 1999). The morphology of *F. japonica* ranges through oval, round, and rectangular shapes (Tomas et al., 2001). Environmental variables (temperature, salinity, pH, and light intensity) on growth are reflected in the morphology and motility of cells (Tomas, 1978; Nakamura & Watanabe, 1983; Khan et al., 1995b, 1998; Marshal & Hallegraeff, 1999). For example, spherical cell morphology in *C. marina* has been associated with extreme temperatures and low salinities, but pH and light intensity did not influence morphology (Khan et al., 1995b; Kahn et al., 1998). In this study, pear-shaped cells of *C. marina* were observed in N-limited and GSe media. In $f/2$ and P-limited media, cells ranged from oval to round. Different media affected growth and cell morphology of *C. marina* and *F. japonica*. In $f/2$ and in GSe media, old *C. marina* cells became narrower and longer, and this was also observed in N-limited cultures. Under these conditions, cells have a greater resemblance to *C. antiqua*. Morphology of *F. japonica* in different culturing media was less polymorphic than *C. marina* cells. It is possible that different cell morphologies are found in the environment as a result of different nutritional and physical conditions. Our observations of cultures confirm morphological variation in raphidopycean species, particularly in size and shape.

It is also of particular importance that the distinctive characteristic of *F. japonica* trichocyst rods concentrated in the posterior part of the cell (Toriumi & Takano, 1973) is almost absent in the strain observed in this study. This unusual characteristic was recently reported for a strain isolated from the Waddenzee in The Netherlands (Tillman & Reckermann, 2002). This variation was explained by the use of artificial seawater culture medium. They support this hypothesis with the observation that a different strain from the same area, cultured in natural seawater, showed a number of large trichocyst. The strain isolated from Bahía Concepción has been maintained in $f/2$ medium prepared with natural seawater for several generations. It is probable that the absence of this feature could be the result of the influence of other factors, such as the nutrient composition of the culture media, permanent availability of nutrients, and/or the absence of competition and predators.

Pigment composition of *C. marina* and *F. japonica* cells cultivated in GSe and $f/2$ media did not change. Pigment signatures in Bahía Concepción have been explained by the presence of several phytoplankton groups: cyanobacteria, diatoms, and dinoflagellates (Bustillos-Guzmán et al., 2000), but Raphidopycean were not considered. Our results suggest that *C. marina* and *F. japonica* contribute to the presence of

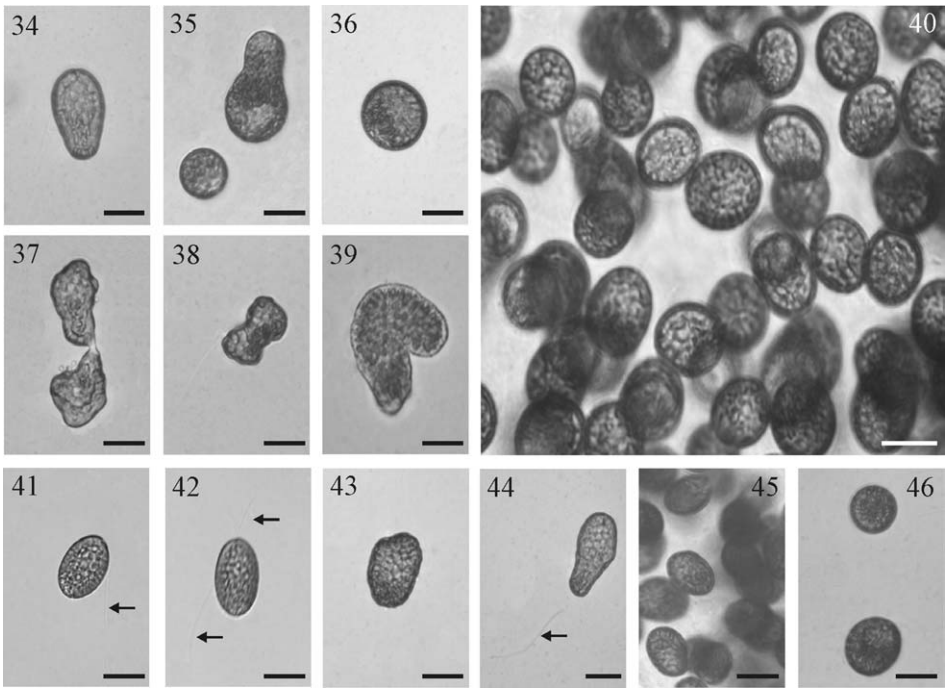


Figures 24–33. Chattonella marina grown in $f/2$ N-limited (24–28), and $f/2$ P-limited (29–33) media. Scale bars = 20 μm. General view of cells in $f/2$ N-limited media (24), spindle cells (25, 26), non-motile spherical cells (27, 28), general view of cells in $f/2$ P-limited media (29), spindle cells (30–32), non-motile spherical cell (33).

several pigments in the bay, mainly chlorophyll *a* and fucoxanthine. Raphidophyceans might contribute to the primary productivity in this bay during summer. Under controlled conditions, *C. antiqua* developed daily vertical migrations and takes up PO_4^{-3} and nitrogen in the nutrient-enriched layer during the night (Watanabe et al., 1991). Additionally, species of this genera form blooms even in rather high concentration of $Si(OH)_4$ (Montani et al., 1989). Migration of the nutrient supply to the surface layer, occurring after stratification, is accompanied by nutrient exhaustion, sinking, and/or inactivation of diatoms. An initial vegetative population, originated by cyst germination, is thought to be essential for bloom formation (Imai et al., 1998). This hypothetical scenario could be linked with summer hydrological conditions prevailing in the central basin of Bahía Concepción, where strong stratification in the summer is characterized by high nutrient concentrations, mainly silicates and phosphates, and anoxia below 20 m depth (Bustillos-Guzmán et al., 2000; Lechuga-Devéze et al., 2001).

Most Raphidophyceae flagellates are common worldwide, but in Mexican marine waters, they had not been detected, mainly because of traditional methods of sampling and preserving marine phytoplankton. These species are very delicate, and deform or disintegrate in formalin solution. Samples preserved with acid lugol solution have different morphology, but can be recognized with a trained eye. Additionally, their importance in routine counts and monitoring has been neglected or underestimated (Loeblich & Fine, 1977). We think that more Raphidophyceae species could be found in Mexico using the proper methods.

Most marine Raphidophyceae are well-known ichthyotoxin producers, more related to reactive oxygen species (ROS), brevetoxins, and polyunsaturated fatty



Figures 34–46. *Fibrocapsa japonica* grown in Gse (34–39), $f/2$ media (40–42), N-limited (43, 44), and $f/2$ P-limited (45, 46) media. Scale bars = 20 μm. Spindle cell (34), pear-shaped and spherical cells (35), spherical cell (36), asexual division (37–39), general view of the cells in $f/2$ media (40), oval cells showing flagella (arrows) (41, 42), oval cell (43), pear-shaped cell with flagella (arrow) (44), general view of cells in $f/2$ P-limited media (45), spherical cells (46).

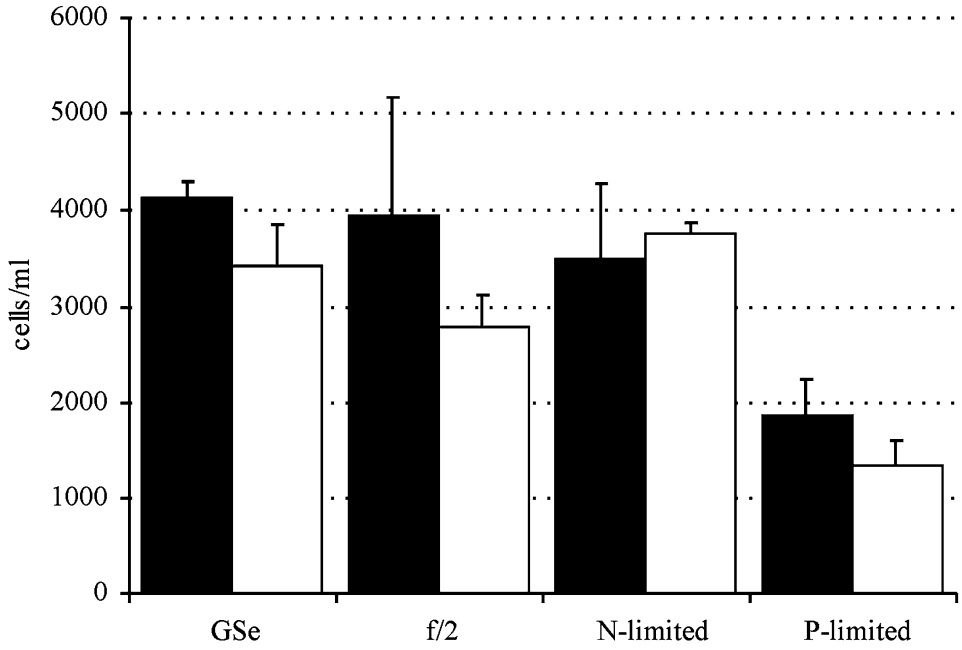


Figure 47. Cell densities of *Fibrocapsa japonica* (full bars) and *Chattonella marina* (empty bars) after 14 days of growth in different media.

acids (Skeen et al., 2002), which cause severe damage to fish gills. Despite this, no reliable reports indicate any mass fish killings in Mexico, although fish culture is not very extensive in Mexico. We plan to isolate more Raphidophycean strains, define their genetic relationships with species of different geographic areas, and determine their toxic qualities, as well as the lethal effects on marine organisms. No toxin analyses have been performed in our strains so far.

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